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siRNAs against the Epstein Barr virus latency replication factor, EBNA1, inhibit its function and growth of EBV-dependent tumor cells

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Abstract

The Epstein Barr virus (EBV) plays a role in maintenance of the tumor phenotype in a number of human cancers. The EBV latency replication factor, EBNA1, is required for persistence of the EBV episome, is anti-apoptotic, and is universally expressed in all EBV-associated tumors. Here, we show that EBNA1-specific siRNAs can inhibit EBNA1 expression and function. siRNAs were generated against three target sites in the EBNA1 messenger RNA, and two of these were found to inhibit EBNA1 expression from an ectopic EBNA1 expression cassette. EBNA1 siRNAs also inhibit endogenously expressed EBNA1 in EBV-positive epithelial and B-cell lines. Using a mini-EBV replication model, siRNA-mediated inhibition of EBNA1 expression suppressed the episomal maintenance function of EBNA1. Lastly, introduction of an EBNA1 siRNA into an EBV-positive tumor cell line inhibited tumor cell growth/survival. These data suggest that siRNAs against EBNA1 may have therapeutic value in EBV-associated diseases.
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Introduction

The Epstein Barr virus (EBV) is a ubiquitous human herpesvirus that establishes a persistent infection and is carried by greater than 90% of the world's population. In immuno-competent individuals, EBV contributes to a number of human cancers including the endemic form of Burkitt's lymphoma (BL), nasopharyngeal carcinoma, Hodgkin's disease, and a small percentage of gastric carcinomas and T cell lymphomas (Kieff, 1996; Miller, 1990; Young and Rowe, 1992). In immuno-compromised individuals, EBV is more problematic, and, despite the use of HAART therapies to treat AIDS patients, there has been no significant change in the incidence of many EBV-associated non-Hodgkin's lymphomas (Buch-binder et al., 1999; Calzolari et al., 1999; Dodd et al., 1992; Green and Eversole, 1989; Jacobson et al., 1999; Ledergerber et al., 1999).

EBV contributes to tumor maintenance and survival through multiple mechanisms including cell cycle promotion, inhibition of apoptosis, and inhibition of inflammatory responses. Much of the cell cycle promoting functions and the anti-apoptotic functions of EBV are conferred through the expression of a limited number of EBV genes that are expressed during the latency phase of the life cycle. Nevertheless, low level reactivation in a small percentage of cells is typically observed in many tumors and may play a role in anti-inflammatory responses since EBV encodes a potent IL10 which is expressed during the lytic replicative cycle (Moore et al., 1990). Furthermore, the immediate early gene product, Zta, was shown to induce TGF-beta1 (Cayrol and Flemington, 1995). Therefore, the contribution of EBV to the tumor phenotype is complex, and any one of these pathways may be potential targets for therapeutics.

From a therapeutic standpoint, an advantageous aspect of virally associated tumors is that they harbor unique genetic material that can theoretically be targeted without effecting normal cell function. Targeting of viral genetic material may be a viable means to specifically block key functions of viruses without influencing regulatory pathways in normal uninfected cells. Although the discovery that siRNA mechanisms are conserved in mammalian systems is still relatively new, there is a significant amount of interest in the possible application of

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siRNA-based strategies for therapeutic purposes. This is due in part to the high level of efficacy observed for siRNA-mediated gene inhibition and in part to the high level of specificity for this approach. Although off-site targeting can occur and must be considered and minimized in any application of siRNA-based usage, the specificity is nevertheless very high relative to many other therapeutic agents.

Although the pattern of latency-associated gene expression varies considerably in different EBV-infected tissues, EBNA1 is unique in that it is universally expressed in all proliferating EBV-infected cells. This strong association with proliferating tissues, including tumor cells, is due partly to its requirement for maintenance of the viral episomal genome during the cell division cycle (Lee et al., 1999; Young et al., 2000). In addition to its fundamental role in maintenance of the viral episome, however, there is evidence that EBNA1 may also make a more direct contribution to the tumor phenotype of EBV-associated tumors. For example, Wilson et al. (1996) have shown that EBNA1 induces B cell neoplasia in transgenic mice. A recent report by Kennedy et al. (2003) showed that EBNA1 has anti-apoptotic properties in most EBV-infected BL tumor lines in tissue culture. For these reasons, EBNA1 may be a particularly attractive target for therapeutic strategies. As mentioned above, the contribution of EBV to the tumor phenotype is complex and likely occurs through the effects of multiple EBV-encoded genes (including lytic cycle associated genes). Targeting EBNA1 function has the potential to impact all of these mechanisms through loss of episomal maintenance as well as impacting tumor survival more directly through the mitigation of EBNA1's tumorigenic/anti-apoptotic activities.

Results

siRNA design

A series of potential EBNA1 siRNA target sites were identified for the B95-8 strain of EBNA1 using a program available online by OligoEngine (www.oligoengine.com). To avoid possible non-specific effects, target sites within the Gly/Ala repeat region of EBNA1 (Fig. 1A) were excluded since this repeat region is highly homologous to human genomic repeat sequences (Heller et al., 1985). Target sites outside of the repeat regions were then blasted, and three target sites (referred to as E1(T1), E1(T2), and E1(T3)) were identified which have minimal homology to human genomic coding sequences. Double-stranded oligonucleotides encoding hairpin structures corresponding to these sites were cloned into the vector, pSUPERretro (Brummelkamp et al., 2002).

Inhibition of ectopic EBNA1 expression

Cotransfection of 1 μg of the pSUPERretro plasmids, E1(T1), E1(T2), and E1(T3) with 1 μg of an EBNA1 expression vector in 100 mm plates containing Hela cells showed that E1(T1) moderately inhibits the expression of EBNA1, while E1(T2) and E1(T3) more effectively inhibit the expression of EBNA1 (Fig. 1B). In an effort to minimize any non-specific effects of the siRNAs mediated through low homology targeting of cellular targets or other activities mediated by double-stranded RNA, titration experiments were carried out to determine the lower end of input siRNA vectors
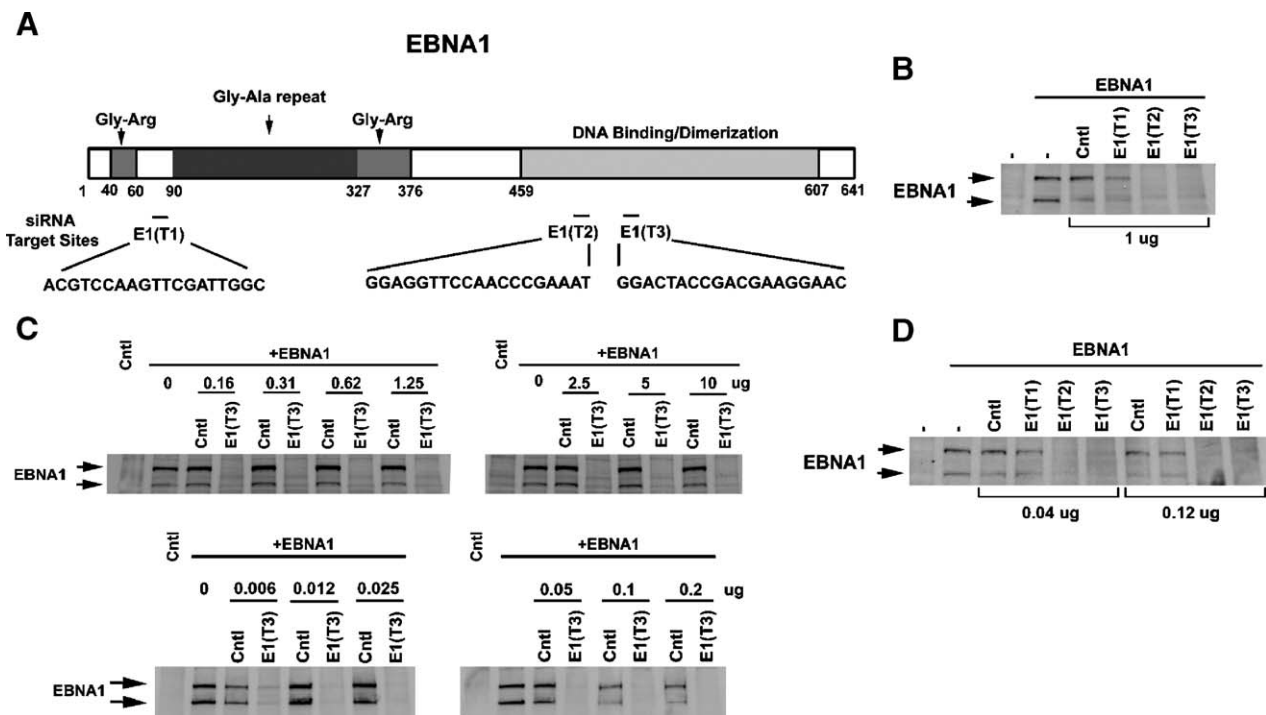

Fig. 1. Analysis of EBNA1 siRNAs for inhibition of EBNA1 expression. (A) Structure of EBNA1 and position and sequence of siRNA target sites. (B–D) One hundred millimeter plates containing Hela cells were transfected with 1 μg of the EBNA1 expression vector, pCEP4, plus the indicated amounts of either pSUPER (cntl), pSUPER—E1(T1), pSUPER—E1(T2), or pSUPER—E1(T3) and analyzed for EBNA1 expression by Western blot analysis 2 days following transfection using a monoclonal anti-EBNA1 antibody [Advance Biotechnologies (catalog no. 13-156-100)].

that still inhibit EBNA1 expression. As shown in Fig. 1C, transfection of as little as 12 ng of the E1(T3) targeting vector (in a 100 mm plate (with 30 μg of carrier DNA)) is sufficient to inhibit EBNA1 expression. E1(T2) appears to be similarly efficient for inhibition of EBNA1 expression, and, as shown in Fig. 1D, 40 ng or 120 ng significantly inhibits EBNA1 expression.

Inhibition of EBNA1 replication function

Next, we used a vector, pCEP, which contains an EBNA1 expression cassette, the EBV origin of replication, and a hygro selection marker as a mini-EBV replication model (Fig. 2A) to assess whether EBNA1 siRNAs are sufficiently active to inhibit EBNA1-mediated replication/episomal maintenance. As shown in Fig. 2B, cotransfection of pCEP with 25 ng, 50 ng, or 100 ng of the control vector, pSUPERretro, had no influence on the number of colonies arising after hygro selection. In

contrast, cotransfection of 25 ng, 50 ng, or 100 ng of E1(T3) with pCEP resulted in a marked reduction in the number of colonies formed. Notably, a few colonies arise which may be due to integration of the pCEP plasmid. To test whether the reduction in colony formation in cells transfected with E1(T3) is due to inhibition of cell growth mediated through possible non-specific effects of the siRNA, cells were cotransfected with the same concentrations of pSUPERretro (cntl) or E1(T3) and the hygro containing plasmid, pBABE-hygro, which does not contain EBNA1 or the EBV origin of replication. Hygro-resistant colonies that arise from these transfections are due specifically to integration of the pBABE-hygro vector and are independent of EBNA1 replication function (note that since integration is a rare event, approximately 5-fold more cells were plated after these transfections than after the pCEP-hygro transfections). As shown in Fig. 2B, similar numbers of colonies are observed in E1(T3) transfections relative to the control (pSUPERretro) transfected plates, indicating that the
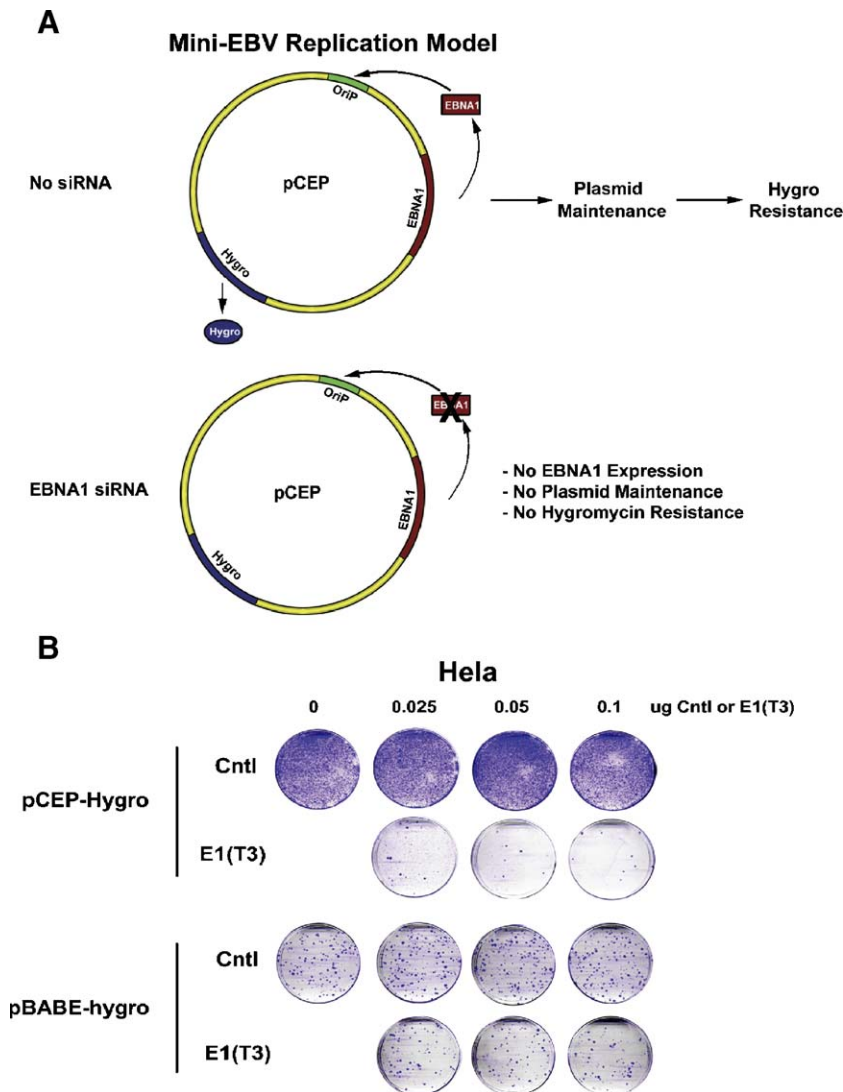

Fig. 2. Inhibition of episomal maintenance function of EBNA1 using mini-EBV replication model. (A) Basis of selection assay. (B) 1 μg of either pCEP (hygro) or pBABE-hygro was cotransfected with the indicated amounts of either pSUPER (cntl) or pSUPER—E1(T3). Two days after transfection, cells were split either 1:10 (for pCEP transfections) or 1:2 (pBABE-hygro transfection) in media containing 250 μg/ml hygromycin. Cells were stained 10–14 days later to visualize colonies.

inhibition of colony formation observed in the pCEP transfections is likely to be specifically due to inhibition of EBNA1 function and not non-specific inhibition of cell growth.

As additional specificity controls, we generated pCEP plasmids that contain conservative mutations in either the T2 or the T3 target sites of EBNA1 (Fig. 3A). While these mutations do not alter the amino acid sequence in the target region, they are sufficient to prevent significant inhibition by their respective siRNA (Fig. 3B) [It should be noted that these mutants were generated using a PCR mutagenesis approach and sequence analysis of the EBNA1 or for each mutant revealed deletions in the Gly/Ala repeat region. These deletions do not affect EBNA1's replication/episomal maintenance function (data not shown and see below), but they do result in alterations in its mobility on Western blots (right panel of Fig. 3B)]. The pCEP (T2) mutant was used to further assess the specificity of the E1(T2) siRNA plasmid in inhibiting EBNA1-mediated replication/plasmid maintenance. As shown in Fig. 3C, while E1(T2) significantly inhibited colony formation of pCEP, it had no impact on colony formation in pCEP(T2mut) transfected cells, further establishing that inhibition of colony

formation in pCEP transfected cells is due to specific inhibition of EBNA1 function and not non-specific effects of the siRNA itself.

Influence of strain variation on siRNA targeting

Since possible strain variation at target sites can influence the ability of siRNAs to target endogenous EBNA1 expression, we amplified a region spanning the T2 and T3 target sites from selected cell lines carried in our laboratory and determined the sequence. The high fidelity polymerase, Pfu, was used, and two separate PCR reactions was carried out for each cell line. In every case, the sequence for both PCR reactions was identical, indicating that the sequence was accurate and not due to PCR errors. In addition, PCR was carried out on a cell line (Boston) that was generated in our laboratory using B95-8 virus, and these sequences matched the published B95-8 sequence. As shown in Fig. 4A, sequence differences were observed in MutuI and Rael cells at both the T2 and the T3 target sites. A previous study has reported changes in the amino acid sequence of EBNA1 from 13 different cell lines
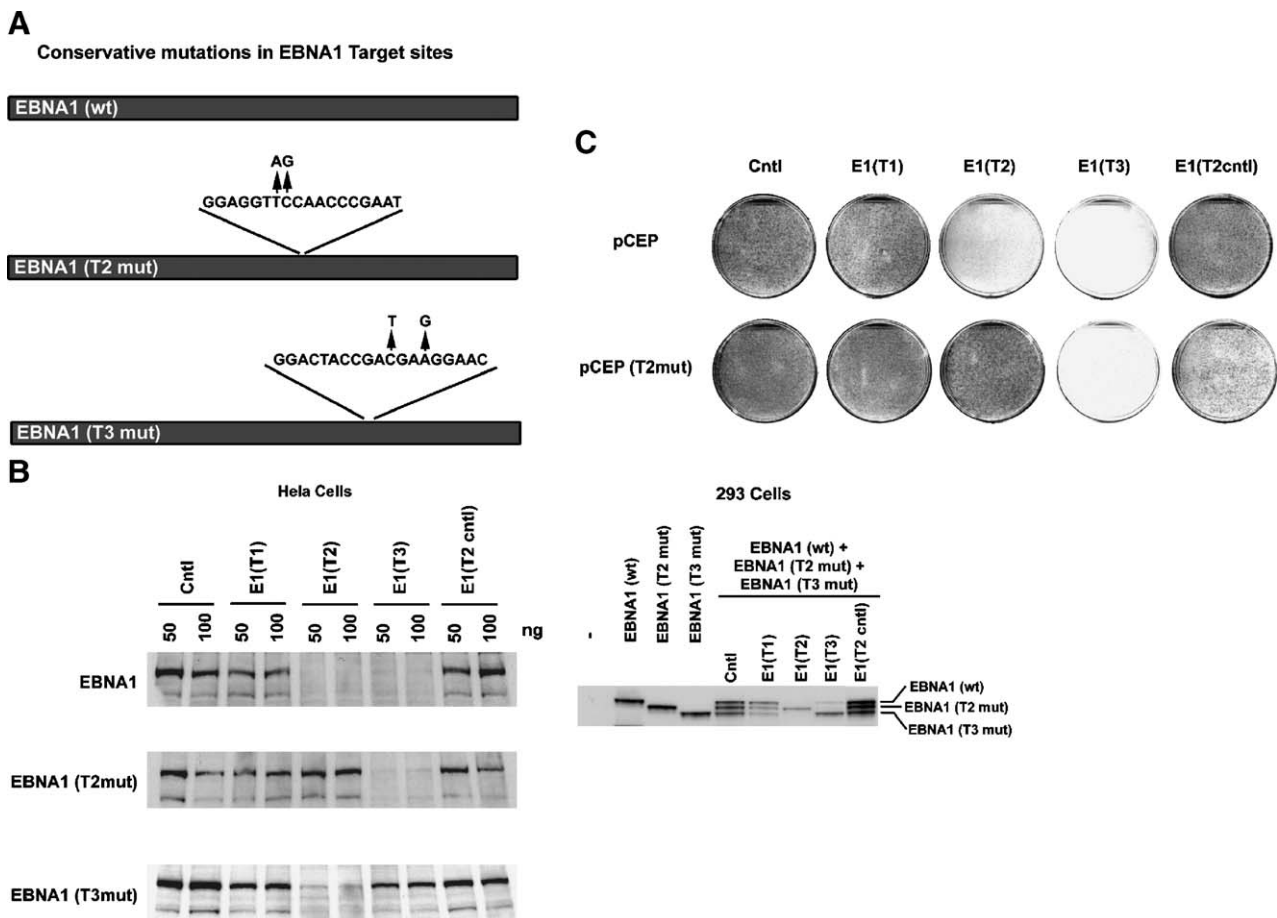

Fig. 3. Conservative mutations in the siRNA target sites of EBNA1 inhibit targeting by E1(T2) and E1(T3) siRNAs and prevent functional inhibition of EBNA1-dependent episomal maintenance function. (A) Illustration of conservative mutations in siRNA target sites of EBNA1. (B) Hela or 293 cells were transfected with the indicated plasmids and assayed for EBNA1, EBNA1 (T2mut), or EBNA1 (T3mut) expression 2 days after transfection by Western blot analysis (using a monoclonal EBNA1 antibody [Advance Biotechnologies (catalog no. 13-156-100)]). [Note: in 293 cells, only the upper EBNA1 form is shown for clarity] (C) Hela cells were transfected with 1 μg of either pCEP or a pCEP plasmid containing the T2 mutation plus 250 ng of the indicated pSUPER-based plasmids. E1(T2cntl) contains a scrambled T2 sequence and serves as an additional negative control.
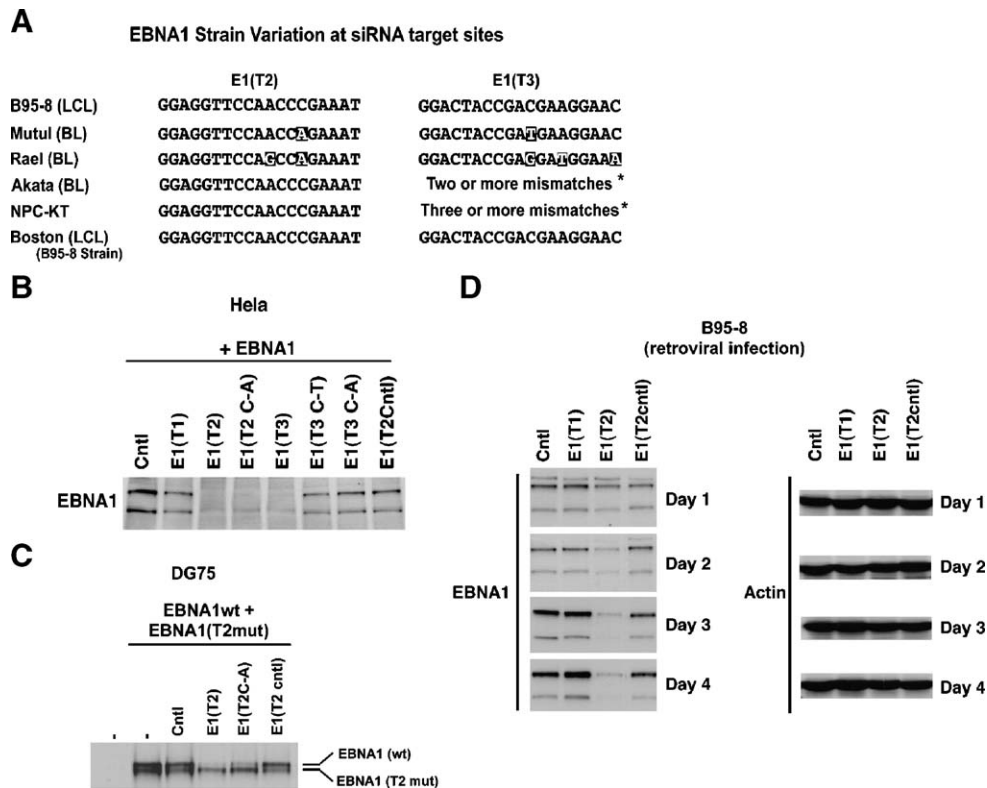
Fig. 4. Analysis of siRNA efficacy with target site strain variants and inhibition of endogenous EBNA1 expression. (A) The T2 and T3 target sites of EBNA1 of MutuI, Rael, and Boston cells were cloned and sequenced. The sequences for Akata and NPC-KT were derived from Chen et al. (note: since only the amino acid sequence was reported in this paper, we cannot discern whether some lines have more than 3 mutations). (B) Hela cells were transfected with 1 μg of pCEP plus 100 ng of the indicated pSUPERretro-based plasmids and assayed for EBNA1 expression by Western blot analysis (Ab). (C) DG75 cells were electroporated with 2 μg of the indicated EBNA1 expression vectors plus 500 ng of the indicated pSUPERretro plasmids by electroporation and assayed for EBNA1 expression 2 days later. For ease of interpretation, only the upper more prominent form of EBNA1 is shown. (D) B95-8 cells were infected with the indicated pSUPERretro siRNA viruses, and puromycin was added 1 day later. Cells were then assayed for EBNA1 expression 1, 2, 3, and 4 days later. Western blot analysis in panels B–F were carried out using a monoclonal antibody against EBNA1 [Advance Biotechnologies (catalog no. 13-156-100)].

(Chen et al., 1999). In this study, 8 out of 13 cell lines were reported to have the B95-8 sequence at the T2 site, while 4 were shown to have the C–A mutation observed in MutuI cells. Considerably more variation was observed at the T3 site with most lines showing at least 2 or 3 differences from the B95-8 sequence.

Three additional derivatives of the T2 and T3 siRNAs were generated in pSUPERretro, one for the T2 site which contains the C–A mutation observed in MutuI cells, one T3 variant which contains the C–T mutation seen in MutuI cells, and another which contains a C–A variant of the same position in T3. To test whether these alterations influence the ability of the T2 or T3 siRNAs to target the B95-8 strain of EBNA1, the T2, T3, or the variants were cotransfected with the B95-8-derived EBNA1 expression vector in Hela cells, and EBNA1 expression was assessed by Western blot analysis (Fig. 4B). While the E1(T2 C–A) variant was able to suppress B95-8 EBNA1 expression, neither of the T3 variants suppressed B95-8 EBNA1 expression. This indicates that, in some cases, a one nucleotide mismatch in an siRNA sequence can still mediate inhibition, while, in others, a one nucleotide mismatch is not tolerated. The E1(T2 C–A) mutant similarly targeted the B95-8 strain of EBNA1 in DG75 cells. Since the T2 site is more conserved and since there is some cross strain targeting at

the T2 site, this may represent a more useful siRNA for possible therapeutic applications.

Inhibition of endogenous EBNA1 expression

To test whether endogenous EBNA1 expression can be inhibited by the T2 siRNA, pSUPERretro, pSUPERretro-E1(T1), pSUPERretro-E1(T2), or a vector containing a scrambled T2 sequence (E1(T2cntl)) was packaged and introduced into B95-8 cells through retroviral transduction. Retroviral transduction was used since introduction of exogenous DNAs into these cells is significantly higher using this method compared to transfection methods. After day one, puro was added to select for successfully infected cells. As shown in Fig. 4D, while E1(T1) and E1(T2cntl) failed to inhibit EBNA1 expression, E1(T2) inhibited EBNA1 expression starting at day 2 and more significantly by day 4. This indicates that endogenous EBNA1 can be specifically inhibited by the E1 (T2) siRNA.

Inhibition of EBV-associated tumor cell growth

The next issue that we wanted to assess was whether E1(T2) can inhibit the survival of EBV-positive tumor cells.

The cell line, NPC-KT, is an EBV-positive epithelial cell line generated through the fusion of a primary nasopharyngeal carcinoma and hypoxanthine-guanine phosphoribosyltransferase (HGPRT)-defective cells derived from adenoid tissues. In NPC-KT cells, long-term persistence of EBV is observed in tissue culture, suggesting that EBV may be required for survival or growth of these cells. Nevertheless, since it is possible that EBV is integrated in these cells and persists simply due to co-segregation with the corresponding chromosome(s), we first tested whether these cells require the function of EBNA1. A dominant negative form of EBNA1 (EBNA1dn) was generated and tested for its ability to inhibit outgrowth of colonies in NPC-KT cells. Either a control plasmid or a plasmid expressing EBNA1dn was transfected into NPC-KT cells and was subjected to selection with puromycin. As shown in Fig. 5A, EBNA1dn inhibited colony formation indicating a survival function for EBNA1 in these cells.

To assess the ability of an EBNA1 siRNA to inhibit the growth or NPC-KT cells, we used a non-vector-based siRNA [i.e. RNA/RNA hybrids (duplex RNA)] to avoid any possible side effects reported previously for hairpin structures (1). Double-stranded RNAs against the T2 site of EBNA1 were first tested for inhibition of ectopic EBNA1 in Hela cells. As shown in Fig. 5B, duplex RNA inhibited EBNA1 expression at concentrations as low as 1 nM. In addition, T2 duplex RNA inhibited endogenous EBNA1 expression in B95-8 and NPC-KT cells (Fig 5B).

To test whether T2 duplex RNA can inhibit growth of NPC-KT cells, T2 duplex RNA or a control siRNA (siGLO) was transfected using lipofectamine 2000, and cells were plated to assess colony formation. As shown in the lower panel of Fig. 5B, fewer colonies were observed in cultures transfected with the T2 duplex RNA vs. control. To determine whether inhibition is specifically due to the EBNA1 inhibitory function or non-specific effects of the siRNA, NPC-KT clones expres-
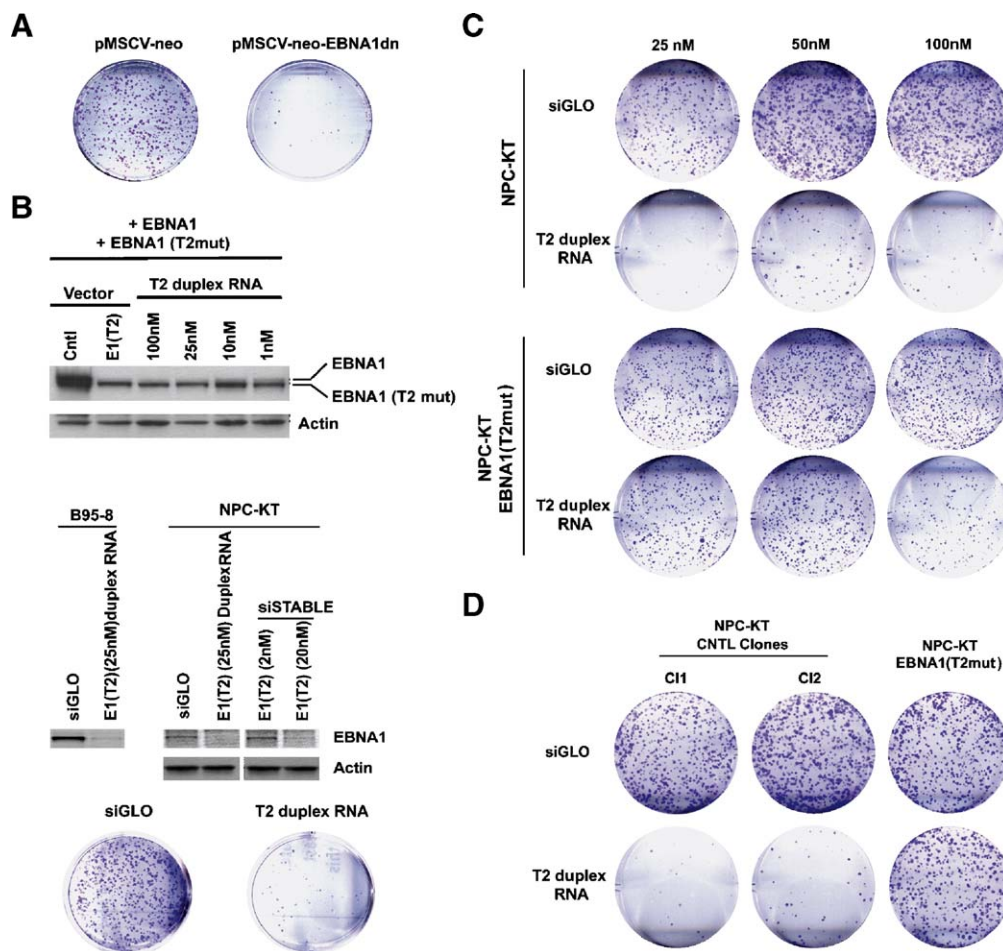

Fig. 5. Inhibition of NPC-KT colony formation by E1(T2) siRNA. (A) NPC-KT cells were transfected with 1 μg of either pMSCV-neo (cntl) or pMSCVneo-E1dn and cultured in 1 mg/ml G418 containing media for 12 days. (B) Hela cells were transfected with EBNA1 and EBNA1 (T2mut) expression vectors plus the indicated amounts of T2 duplex siRNAs (upper panel). B95-8 and NPC-KT cells were transfected with the indicated amounts of either duplex T2 RNA or siSTABLE T2 RNA (ordered from Dharmacon) (middle panel). Endogenous EBNA1 in NPC-KT cells was detected using a human serum reactive to NPC-KT EBNA1. Lower panel: NPC-KT cells were transfected with 50 nM T2 duplex RNA or 50 nM siGLO in four successive transfections (at a 2-day intervals). Cells were then plated to assess colony formation. (C) NPC-KT cells and a stable NPC-KT EBNA1 (T2mut) clone were transfected with T2 duplex RNA or the control siRNA, siGLO. Four transfections were carried out at a 2-day interval, and cells were plated to assess colony formation. (D) NPC-KT control clones, Cl1 and Cl2, or the NPC-KT EBNA1(T2mut) clone was transfected in three successive transfections with 100 nM duplex T2 RNA or the control siRNA, siGLO. Cells were then plated for colony formation.

sing EBNA1(T2mut) were generated. As shown in Fig. 5C, while T2 duplex RNA inhibits colony formation of the parental NPC-KT cells, there is little inhibition of an NPC-KT clone expressing the EBNA1(T2mut). Furthermore, T2 siRNA inhibits colony formation of two NPC-KT clones containing the EBNA1(T2mut) control plasmid but not NPC-KT EBNA1(T2mut) cells (Fig. 5D). Therefore, the observed inhibition of NPC-KT cell growth is due to inhibition of EBNA1 function and not secondary effects of the siRNA.

Discussion

Previous studies have shown that siRNAs directed against RNA viruses including human immunodeficiency virus (HIV), hepatitis C virus (HCV), and influenza virus may have potential therapeutic value in inhibiting virus propagation (Ge et al., 2003; Jacque et al., 2002; Kapadia et al., 2003; Novina et al., 2002; Randall et al., 2003). The use of siRNA technology to inhibit pathologies associated with DNA viruses requires specific targeting of the mRNA of genes that are required for key viral functions. Several reports have shown that targeting the human papilloma virus (HPV)-encoded oncogenes, E6 and E7, may be a viable means of inhibiting the survival of HPV-associated malignancies (Butz et al., 2003; Hall and Alexander, 2003; Jiang and Milner, 2005). In our study, we have developed siRNAs against the EBV-latency-associated gene, EBNA1, which is ubiquitously expressed in all proliferating EBV-associated tissues. Since EBNA1 is required for maintenance of the viral episome, inhibition of EBNA1 function may ultimately lead to the loss of viral genomes with the concomitant loss of cell cycle promoting functions, anti-apoptotic functions, and anti-inflammatory functions of other EBV-encoded gene products. In addition, EBNA1 itself has been shown to promote tumorigenesis and to provide an anti-apoptotic response in B cell tumors. Therefore, EBNA1 may be an effective target in therapeutic strategies against EBV-associated malignancies.

A recent report has shown that adenoviral delivery of a dominant negative form of EBNA1 into EBV-associated tumor cells effectively inhibits tumor growth (Nasimuzzaman et al., 2005), supporting the contention that blocking EBNA1 function may ultimately be a viable strategy for treating EBV-associated tumors. The use of EBNA1 siRNAs may have some advantages over dominant negative forms of EBNA1 in that they cannot only be delivered through viral transduction but can also be delivered directly using duplex RNAs. The use of duplex siRNAs avoids some of the immunological reactivity problems associated with adenoviral vectors, and the continued development of modifications to siRNAs which enhance stability and efficacy may ultimately make siRNAs a preferred method for inhibiting EBNA1 function.

We have identified two siRNAs target sites in the EBNA1 RNA that are effective in inhibiting EBNA1 expression and replication function, and we have shown that one of these sites (T2) can inhibit tumor cell growth in an EBV-positive tumor cell line. While the functional inhibition experiments were carried out using cells containing virus with the B95-8 T2 sequence, it is also likely that strains carrying the other major T2 genotype (the C–A variant) may similarly be effectively targeted using siRNA containing this variant sequence. In addition, changes at this position can be tolerated without abolishing the ability of an siRNA to target EBNA1 expression so even single siRNAs may be able to target multiple viral strains. At the T3 site, there appears to be a higher level of restriction for strain differences observed at position 11. Changing the cytosine at position 11 to either a T or an A abolished the ability of the B95-8 T3 siRNA to target EBNA1. Therefore, targeting EBNA1 through the T3 site will require the use of siRNAs with the appropriate siRNA variant sequence.

In conclusion, we have shown that EBNA1-specific siRNAs can inhibit EBNA1 function and that they can inhibit the growth/survival of an EBV-associated cell line that requires EBNA1 function in tissue culture. These data support the idea that EBNA1-specific siRNAs may have potential therapeutic value in treating EBV-associated tumors, and we believe that this line of investigation warrants further investigation.

Materials and methods

Cell culture

Hela, 293, and NPC-KT cell lines were cultured in Dulbecco's Modified Eagles Medium (DMEM) containing 10% fetal bovine serum (FBS). DG75, MutuI, Rael, and B95-8 cells were cultured in RPMI plus 10% fetal bovine serum. Drug selections were carried out using either 250 μg/ml hygromycin, 1 μg/ml puromycin, or 1 mg/ml G418.

Electroporation

Cultures were grown to near saturation in RPMI (+10% FBS, +Pen/Strep). The day before electroporation, an equal volume of fresh media [RPMI (+10% FBS, +Pen/Strep)] was added. Cells were counted, spun down, and 3×10^7 cells were suspended in 0.4 ml RPMI for each sample. A total of 30 μg of DNA was added including the indicated amounts of plasmid plus carrier DNA (the plasmid, pGL3basic). Electroporations were carried out using a BTX Electrocell Manipulator, ECM600 using the following conditions: voltage = 265, capacitance = 975 μF, resistance = 720 Ω. After discharge, 0.6 ml of RPMI was added, and cell suspension was immediately pipetted up and down several times and transferred to a T25 flask containing 10 ml RPMI (+10% FBS, +Pen/Strep). Cells were cultured for 48 h prior to harvesting.

Calcium phosphate transfection

A modified calcium phosphate precipitation method was used for transfections. Briefly, cells were plated the day before transfection onto 100 mm tissue culture plates. The next morning, the media was replaced with 8 ml of DME containing 10% FBS. 6–8 h later, DNA precipitates were formed using the indicated plasmids plus the carrier plasmid, pGL3basic (for a total amount of 30 μg DNA). DNAs were added to 500 μl 1×

HBS (21 mM HEPES (acid), 137 mM NaCL, 5 mM Dextrose, 50 mM KCl, 0.7 mM Na_2HPO_4, pH 7.1). Thirty microliters of 2.5 M $CaCl_2$ was then added, and the samples were mixed immediately. After 20 min at room temperature, precipitates were added dropwise to tissue culture plates. 16–20 h later, the media was replaced with fresh DME (+10% FBS, +Pen/Strep) 1× HBS.

Duplex RNA transfections

siRNAs were introduced into cells using lipofectamine 2000 (InVitrogen, Cat. #11668-019). Transfections were carried out essentially as described in the manufacturer's instructions with minor modifications. Briefly, 1 day before transfection, 2×10^5 cells were plated into each well of a 6-well plate in 2 ml DME (+10% FBS, +Pen/Strep). Immediately before transfection, the media was replaced with 1.5 ml of fresh DME (without FBS or Pen/Strep). Five microliters lipofectamine 2000 was diluted in 250 µl DME (without FBS or Pen/Strep) for each transfection and mixed with the indicated amounts of siRNAs (diluted in 250 µl serum and antibiotic-free DME). Mixtures were incubated for 20 min at room temperature prior to the dropwise addition to cells. The cells were kept in transfection medium for 8 h before media was replace with fresh DME (+10% FBS, +Pen/Strep). Forty eight hours later, the cells were split for either subsequent transfection, Western blot analysis, or cell colony assays.

Retroviral infection

293 cells were cotransfected with 10 µg of retroviral plasmid, 10 µg each of the packaging plasmids, VPACK-GPdI and pCI-VSV-G (generous gift of Dr. Brian Schaefer) using calcium phosphate transfection. The next morning, the media was replaced with 10 ml of fresh DME (+10% FBS). Twenty four hours later, viral supernatants were taken, spun 2× at 500 × g to precipitate floating 293 cells, and used for infections. B95-8 cells were spun down and suspended in DME (+10% FBS) containing 16 µg/ml polybrene, and 1 ml was added to each well of a 6-well plate. One milliliter of viral supernatant was then added to each well of 6-well plate and mixed. Plates were incubated for 1 h in 37 °C incubator, spun for 1 h at 1000 × g, and then incubated again in a 37 °C incubator for 3 h. Cells from 6 wells were then collected, spun down, suspended in 12 ml RPMI (+10% FBS), and placed in 37 °C 5% CO_2 incubator.

Western blot analysis

Cells were spun down, washed one time with 1× PBS, and suspended in 15 pellet volumes of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading (Laemmli) buffer (Maniatis et al., 1982). Samples were then boiled for 20 min to shear DNA, and lysates were subjected to SDS-PAGE separation and transferred to nitrocellulose membranes. The blots were blocked for 30 min in Tris-buffered saline containing 5% low-fat powdered mild and 1% fetal bovine serum and then incubated with the indicated primary antibody (in blocking buffer) for either 1 h at room temperature or

overnight at 4 °C. The blots were washed once with 1× PBS, once with 1× PBS buffer containing 0.5% (vol/vol) Tween 20, and twice with 1× PBS buffer alone (each was carried out for approximately 15 min). The blots were then incubated with peroxidase-conjugated secondary antibody in blocking buffer for 1 to 2 h at room temperature. Blots were washed as described above and analyzed with an enhanced chemiluminescence detection system (Amersham) according to manufacturer's recommendations, and filters were exposed to Kodak XR film. Antibodies used are indicated in respective figure legends.

PCR and sequencing

The ultra high fidelity PCR system from Stratagene (Cat #600166) was used to amplify the EBNA1 T2/T3 region from MutuI, Rael, and Boston cells using the primers, left primer: 5′-AAGGAGGGTGGTTTGGAAAG-3′, and right primer (Boston, B95-8): 5′-TGGAATAGCAAGGGCAGTTC-3′, right primer (Mutu, Rael): 5′-TGGAATAGCAAGGCCAATTCC-3′. PCR reactions were carried out using the following conditions: denaturation—95 °C (30 s), annealing—62 °C (30 s), and extension—72 °C (30 s), 20 cycles. PCR products were purified using the QIAquick gel extraction kit (Qiagen) and sequenced.

Cloning

The E1(T1), E1(T2), and E1(T3) siRNA vectors were generated by cloning synthetic double-stranded 64-mer oligo-nucleotides (designed based on program on the web site, www.oligoengine.com) containing HindIII and BglII over-hangs into pSUPER and pSUPERretro. Cloning into HindIII and BglII digested pSUPERretro proved difficult since the HindIII and BglII sites are adjacent to each, making digestion with both enzymes a rare event. Oligos were therefore cloned into BglII and XhoI cut pSUPER and pSUPERretro with the addition of a HindIII/XhoI adaptor from eZclone Systems, LLC (New Orleans). All other siRNAs were designed with BglII and XhoI overhangs and cloned directly into BglII and XhoI cut vectors. All clones were sequenced, and clones with no errors were used for analysis. Site-directed mutagenesis of EBNA1 was carried out using the Stratagene QuickChange site-directed mutagenesis kit according to manufacturer's recommendations.

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